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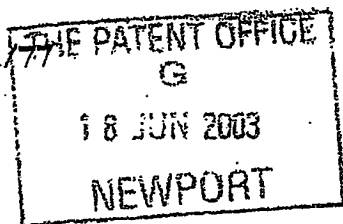
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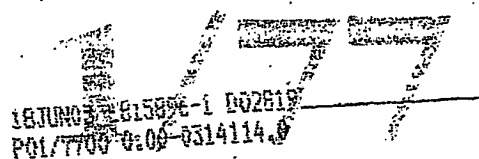
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Keith W Nash & Co  
90-92 Regent Street  
Cambridge  
CB2 1DP

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C1397.00/M

**Title:**        Screening Assay

### **Field of the Invention**

The present invention relates to a method of screening a test substance for possession of binding activity for MSP1<sub>42</sub>. In preferred embodiments the invention provides a method of screening a test substance to identify those which have the ability to inhibit or otherwise interfere with the natural protease-mediated processing of MSP1<sub>42</sub> into MSP1<sub>33</sub> and MSP1<sub>19</sub> fragments.

### **Background of the Invention**

The protozoon *Plasmodium falciparum* develops and replicates within erythrocytes, releasing merozoites that invade new red blood cells. This stage of the parasite's life cycle is responsible for the disease malaria, and inhibition of merozoite invasion reduces parasitaemia, with beneficial outcome for the host.

Several proteins have been identified on the surface of the merozoite (Holder, 1994 Parasitology 108, Suppl. S5-18). For example, *P. falciparum* MSP1 (merozoite surface protein 1) is synthesised as a ~200 kDa precursor and is present on the surface of the late stage parasite within the erythrocyte. At or immediately prior to merozoite release, MSP1 is cleaved (primary processing) into four fragments that form part of a protein complex on the surface of the free merozoite. One of these fragments, the C-terminal 42 kDa-polypeptide (MSP1<sub>42</sub>), has a GPI-anchor holding the complex to the parasite surface. At erythrocyte invasion the protein complex is released from the merozoite surface following secondary processing involving a single proteolytic cleavage within MSP1<sub>42</sub>. The 33 kDa N-terminal part of MSP1<sub>42</sub> is shed with the complex whereas MSP1<sub>19</sub>, the C-terminal part of MSP1<sub>42</sub>, remains on the surface of the invading merozoite. MSP1<sub>19</sub> contains two epidermal growth factor (EGF) domains (Morgan *et al*, 1999 J. Mol. Biol. 289, 113-122). Certain MSP1<sub>19</sub>-specific monoclonal antibodies (mAbs) inhibit both secondary processing

and erythrocyte invasion (Blackman *et al*, 1994 J. Exp. Med. 180, 389-393) suggesting that inhibitors of the protease responsible for MSP1 secondary processing inhibit invasion. Prevention of secondary processing of MSP1 may thus be a good chemotherapeutic target.

Suramin is a polysulfonated naphthyl urea that has been used for many years as a trypanocide in the treatment of sleeping sickness and more recently as a treatment for filariasis (Hawking 1978 Adv. Pharmacol. Chemother. 15, 289-322). Suramin is a symmetrical molecule and highly negatively charged. The structure of the compound is illustrated schematically in Figure 1. It has multiple biological effects *in vivo*, reviewed by, for example, Scher & Kelly (PPO Updates 1993 7, 1-16).

In addition to the clinical usage noted above, suramin has been the subject of several clinical trials because of reported antiviral and antitumour effects. However, suramin is not widely used therapeutically because it has a narrow therapeutic window, being highly toxic and associated with unpleasant undesirable side effects (Voogol *et al*, 1993 Pharmacol. Rev. 45, 177-203).

In addition to suramin *per se*, numerous analogues of suramin have been synthesised, some of which have also been the subject of clinical trials, in an attempt to find substances which have the efficacy of suramin but with less toxicity. Many suramin analogues have been disclosed and described by Firsching-Hauk *et al*, (2000 Anti-Cancer Drugs 11, 69-77) and by Dhar *et al*, (2000 European Journal of Cancer 36, 803-809).

There is one report (Dluzewski *et al*, "Inhibition of Malaria Invasion by Extracellular ATP Analogues"; in Molecular Approaches to Malaria, 2<sup>nd</sup>-5<sup>th</sup> Feb. 2000, Erskine House, Lorne, Victoria, Australia, Ed. M. Macreadie, ISBN 0 646 38983 1) that suramin inhibits merozoite invasion of erythrocytes, although the mechanism was unknown and not disclosed.

### Summary of the Invention

In a first aspect the invention provides a method of screening a test substance for possession of binding activity for MSP1<sub>42</sub> or a fragment thereof, the method comprising the steps of: combining or contacting, in any order,

- (i) a molecule comprising MSP1<sub>42</sub> or a fragment thereof,
- (ii) the test substance, and
- (iii) a comparison substance known to have binding activity for MSP1<sub>42</sub> or a fragment thereof;

and determining the presence and/or amount, if any, of comparison substance and/or test substance bound to the MSP1<sub>42</sub> or fragment thereof. The method may provide results in either a qualitative or a quantitative manner. Not only can the method of the invention be used to identify compounds which are able to bind to MSP1<sub>42</sub> or a fragment thereof, but may alternatively be used to characterise such binding (e.g. measurement of absolute or relative binding affinity; or identification of the binding site on MSP1<sub>42</sub> - by using different fragments or variants of MSP1<sub>42</sub>).

The MSP1<sub>42</sub> used in the method of the invention may be, for example, derived from Plasmodium parasites and purified or, more preferably, may be recombinant MSP1<sub>42</sub> expressed in, for example, a bacterial, yeast or eukaryotic cell culture in the absence of Plasmodium parasites. The MSP1<sub>42</sub> may, in theory, be present as part of a larger molecule (e.g. the MSP1 200kDa precursor molecule) but this is not preferred as the possibility of binding of the test substance to the MSP molecule outside the MSP1<sub>42</sub> portion must be prevented or excluded from consideration in some way. Accordingly, it is preferred that the molecule used in the method of the invention is substantially limited to MSP1<sub>42</sub> or a fragment thereof, and any other portions of the molecule are restricted to components which are known not to bind to the test substance. Nevertheless, additional components may be desirable to include in, or attach to, the MSP1<sub>42</sub> molecule e.g. to facilitate immobilisation to a solid surface such as the well of a microtitre plate or other substrate. If deemed appropriate, any additional component included in or attached to the MSP1<sub>42</sub> molecule could be separately tested for binding to the test substance, so as to exclude such a possibility or allow for such binding in consideration of the assay results.

The method of the present invention also allows for use of a fragment of MSP1<sub>42</sub>. The fragment will preferably comprise at least 50 amino acids, more preferably at least 100 amino acids, and most preferably at least 150 amino acids. Desirably the fragment comprises one of the naturally-occurring fragments derived by protease degradation: MSP1<sub>19</sub> and MSP1<sub>33</sub>. The MSP1<sub>19</sub> fragment is derived from the C terminal portion of MSP1<sub>42</sub> and the MSP1<sub>33</sub> fragment is derived from the N terminal portion of MSP1<sub>42</sub>.

The MSP1<sub>42</sub> molecule or fragment thereof may be a naturally-occurring molecule (that is, comprise an amino acid sequence identical to that encoded by a Plasmodium organism) or may be slightly different (e.g. comprise a few say, less than 20, amino acid residue substitutions, preferably less than 10 substitutions) relative to a naturally-occurring molecule. Thus, for example, the MSP1<sub>42</sub> molecule or fragment thereof may be a mutant of a naturally-occurring wild type sequence. The mutant may be a spontaneous mutant or a laboratory-induced mutant. The MSP1<sub>42</sub> molecule or fragment thereof may also comprise substitutions and/or additional residues e.g. to facilitate expression or purification.

If desired, the test substance can be assayed or screened against a number of different MSP1<sub>42</sub> molecules (or fragments thereof) so as to provide extra information e.g. about the binding site and/or other properties of the interaction with MSP1<sub>42</sub> or the fragment thereof.

The MSP1<sub>42</sub> molecule or fragment thereof may be derived from, correspond to or be similar to that obtainable from any Plasmodium species including, for example, *P. cynomolgi*, *P. knowlesi*, *P. berghei*, *P. chabaudi*, or *P. yoelii*. It is preferred, however, that the MSP1<sub>42</sub> molecule or fragment thereof may be derived from, correspond to or be similar to that obtainable from one of the Plasmodium species known to be capable of infecting humans i.e. *P. falciparum*, *P. vivax*, *P. malariae* and *P. ovale*. Of these, *falciparum* and *vivax* are preferred.

The test substance may be any substance of potential interest. Preferably the test substance is a compound or mixture of potential therapeutic interest and is therefore preferably of low toxicity for mammals. In particular the test substance may form part of a library of substances, e.g. a library produced by combinatorial chemistry, or a phage display library.

The general principle of the method of the invention is that of a competition assay – if the test substance is able to bind to MSP1<sub>42</sub> or a fragment thereof, and more especially if it binds at or close to the same site as that bound by the comparison substance, then the presence of the test substance will compete with, interfere or inhibit the binding of the comparison substance to the MSP1<sub>42</sub> molecule or fragment thereof.

The binding affinity of the test and comparison substances may be quite different, and it may be desirable therefore to perform the method using a variety of ratios of test and comparison substance concentrations. The method may be performed by contacting the test and comparison substances substantially simultaneously with the MSP1<sub>42</sub> molecule or fragment thereof. Alternatively, one of the substances may be pre-incubated with the MSP1<sub>42</sub> molecule or fragment thereof, and the other substance introduced subsequently to see if any displacement of pre-bound test or comparison substance, as appropriate, takes place.

The step of determining the amount of bound test and/or comparison substance may be achieved using any of the numerous suitable assay techniques known to those skilled in the art, such as radioassay, fluorescence assay, ELISA, isothermal titration calorimetry (ITC), surface plasmon resonance (SPR) and the like. The assay is preferably one which is amenable to automation and/or high throughput screening. Desirably the assay is performed on a disposable solid support such as a microtitre plate or similar.

The comparison substance may be any substance which is known to bind to MSP1<sub>42</sub>. The comparison substance may be, for example, an antibody or antigen-binding variant thereof (such as an Fab, Fv, scFv etc), a peptide or synthetic chemical compound. The comparison substance may conveniently be labelled with a readily detectable marker,



which serves to facilitate detection of the labelled comparison substance and hence determination of the amount bound (or unbound) to the MSP1<sub>42</sub> molecule or fragment thereof, although this is not essential. The label may comprise, for instance, a radio label, an enzyme label, an antibody label, a fluorescent label, a particulate (e.g. latex) label or the like.

The present inventors have identified substances which, upon binding to MSP1<sub>42</sub>, inhibit the normal protease-mediated processing of the molecule into MSP1<sub>33</sub> and MSP1<sub>19</sub> fragments, which processing is an essential part of the pathway by which Plasmodium merozoites invade erythrocytes. Thus, by using such a substance as the comparison substance, the present invention provides a method of identifying test substances which will bind to the same or similar portion of the MSP1<sub>42</sub> molecule and hence should similarly inhibit processing of MSP1<sub>42</sub> into MSP1<sub>33</sub> and MSP1<sub>19</sub> and/or inhibit merozoite invasion of erythrocytes. Accordingly in preferred embodiments the comparison substance is a substance which, upon binding to MSP1<sub>42</sub>, inhibits the normal protease-mediated processing of the molecule into MSP1<sub>33</sub> and MSP1<sub>19</sub> fragments. In such an embodiment the method of the invention provides, in effect, a method of screening a test substance for the ability to interfere with or inhibit secondary processing of MSP1<sub>42</sub> and/or inhibit merozoite invasion of erythrocytes. In this way the method of the invention can be used to identify/screen drug-like compounds with potential application as anti-malarials and which are less toxic than suramin.

One such group of substances is constituted by suramin and various analogues thereof. The structure of suramin is shown in Figure 1. The inventors have found that suramin and analogues thereof bind to MSP1<sub>42</sub> or fragments thereof derived from, *inter alia*, *P. falciparum*, *P. vivax* and *P. yoelii*.

The symmetrical nature of the suramin molecule means that some analogues comprise structural variants based on just ½ of the suramin molecule (comprising 4 aromatic rings and half of the anionic substituents). Such analogues may comprise just one or two sulphonyl groups (instead of the three sulphonyl groups present on ½ the suramin

molecule). The sulphonyl groups may be present at different positions to those that they occupy in suramin. Alternatively, anionic, cationic, or uncharged groups may be present instead of, or in addition to, the sulphonyl groups. The same comments apply to variants which are structural analogues of the whole suramin molecule.

Accordingly, the term "suramin analogue", for the purposes of the present invention, encompasses all molecules with at least two aromatic ring(s) the rings comprising, between them, at least one, preferably at least two, and more preferably at least three, uncharged or charged (preferably negatively charged) substituent groups, and which are able to bind to MSP1<sub>42</sub> or a fragment thereof with an affinity of at least 5 $\mu$ M.

The substituent group(s) attached to the aromatic ring are preferably negatively charged under the conditions in which the assay method of the invention is performed. The substituent group may be a sulphonyl group (which is preferred), as in the suramin molecule, or may be any other suitable group of generally similar size and charge.

The aromatic rings may be directly adjacent (i.e. having two carbon atoms common to each of two rings) or may be separated by intervening atoms.

Preferably the two or more aromatic rings will be covalently bonded to urea or a urea derivative comprising the moiety  $\text{-NHCONH-}$ , such as m-aminobenzoyl ( $\text{-NHCO}$



In preferred embodiments the suramin analogue comprises two or more aromatic rings, with negatively charged substituents, attached (directly or indirectly) to one end of a molecule of urea or a urea derivative, and two or more aromatic rings, with negatively charged substituents, attached (directly or indirectly) to the other end of the molecule of urea or urea derivative. Compounds of the type shown in groups B and C of Table 1 represent examples of preferred suramin analogues.

Suramin and many of its analogues possess intrinsic fluorescence. Accordingly, it is possible to determine binding of suramin to e.g. MSP1<sub>42</sub> by measuring the amount of intrinsic suramin-mediated fluorescence in an assay system. Alternatively, the suramin or suramin analogue may be labelled with a conventional label moiety and the presence and/or amount of binding determined by detection and/or measurement of the conventional label.

The present inventors have found that suramin binds relatively tightly to MSP1<sub>42</sub> and MSP1<sub>33</sub>, but relatively weakly to MSP1<sub>19</sub>. They have further identified particular residues in MSP1<sub>19</sub> which may be involved in suramin binding.

Figure 8 shows alignment of the amino acid sequences of MSP1<sub>42</sub> protein from various *Plasmodium* sp.

In Figure 8 Pfwel, PfMAD, CAMP, UPA, FC27 and 3D7 are all *P. falciparum* strains; PV1 and PV2 are *P. vivax* strains; Pcyn is *P. cynomolgi*; PK is *P. knowlesi*; Py is *P. yoelii*; Pb is *P. berghei* and Pc is *P. chabaudi*. The table below sets out the accession numbers in the GenBank/EMBL/DDJB databases of the various amino acid sequences.

Parasite species	Parasite line	Sequence accession number
<i>Plasmodium falciparum</i>	Wellcome	X02919
<i>Plasmodium falciparum</i>	MAD 20	X05624
<i>Plasmodium falciparum</i>	CAMP	X03831
<i>Plasmodium falciparum</i>	Uganda- Palo Alto (UPA)	M37213
<i>Plasmodium falciparum</i>	FC27	M19143
<i>Plasmodium falciparum</i>	3D7	Z35327
<i>Plasmodium falciparum</i>	Belem	AF435594
<i>Plasmodium vivax</i>	Sal-1	M75674
<i>Plasmodium vivax</i>	Ceylonesis	U25743
<i>Plasmodium cynomolgi</i>	Nuri	X91855
<i>Plasmodium knowlesi</i>	YM	J04668
<i>Plasmodium yoelii</i>	K173	U43521
<i>Plasmodium berghei</i>	IPP	M34947
<i>Plasmodium chabaudi</i>		

The cleavage site between MSP1<sub>33</sub> and MSP1<sub>19</sub> is, in the alignment, between residues 316 and 317 and is indicated by a jagged arrow, such that residue 317 of MSP1<sub>42</sub> corresponds to residue 1 of MSP1<sub>9</sub>. The shading represents various blocks of conserved sequence.

The inventors have found, in particular, that the following residues of MSP1<sub>9</sub> of *P. falciparum* are implicated in interaction with suramin: I2, H5, F19, H21, L22 and R25. Significantly, H5 is conserved across all species, F19 is semi-conserved (also appearing as Y19) and L22 is widely conserved. Accordingly, it is likely that substances (either test substances or comparison substances) which interact with at least one, two, three, four, five or six (in increasing order of preference) of the MSP1<sub>19</sub> residues identified above will similarly inhibit MSP1<sub>42</sub> processing and/or merozoite invasion of erythrocytes.

The assay may involve simple detection and/or measurement of comparison and/or test substance bound to the MSP1<sub>42</sub> molecule or fragment thereof (in a "direct" binding assay), but such binding may be detected indirectly e.g. by determining any inhibitory effect on merozoite invasion of erythrocytes and/or inhibition of MSP1<sub>42</sub> processing.

Methods similar to those used by the inventors and described in the present specification may, with the benefit of the present disclosure, be employed without undue inventive effort by those skilled in the art to identify similar residues in the MSP1<sub>33</sub> fragment of MSP1<sub>42</sub> which are involved in binding to suramin or suramin analogues and, based on knowledge of the 3 dimensional structure of suramin and MSP1<sub>42</sub>, this information could be used to computer model compounds which will be predicted to bind to MSP1<sub>42</sub> and may therefore inhibit MSP1<sub>42</sub> processing.

Given the highly hydrophobic nature of the suramin molecule, the inventors propose that the residues of MSP1<sub>33</sub> involved in binding to suramin are likely to be hydrophobic, so as to form a hydrophobic pocket or cleft into which the suramin molecule can become inserted. Since the inventors have found that MSP1<sub>42</sub> from both *P. falciparum* and *P. vivax* can bind suramin, it also seems reasonable to suppose that the residues will be at least semi-conserved between these species.

In a second aspect the invention provides for a method of preventing and/or treating malarial disease by administering an effective amount of suramin or, more preferably, a suramin analogue, to a mammalian (preferably human) subject in need of such treatment. More specifically the invention provides for a method of inhibiting merozoite invasion of erythrocytes in a mammalian host.

In a third aspect the invention provides for use of suramin or, more preferably, a suramin analogue, in the preparation of a medicament to prevent and/or treat malarial disease in a mammalian subject.

Preferred suramin analogues are those compounds which exhibit a degree of activity similar to that of suramin in terms of inhibiting MSP1<sub>42</sub> processing and/or inhibiting merozoite invasion of erythrocytes, but with reduced toxicity for the mammalian subject.

The invention will now be described further by way of illustrative example and with reference to the accompanying drawings, in which:

Figure 1 is a schematic representation of the structure of suramin;

Figure 2 is a graph showing % relative parasite growth in the presence of suramin or NTS at various concentrations;

Figures 3A and 3B are pictures of Western blots of MSP1 proteins subjected to various treatments;

Figure 4 is a graph showing fluorescence intensity (arbitrary units) for suramin at various concentrations interacting with MSP1;

Figures 5A and B are plots of <sup>1</sup>H chemical shift change (in ppm) against suramin concentration (in mM) for residues H5 and L22 respectively of MSP1<sub>19</sub>;

Figure 6(i) is a representation of the 3D structure of MSP1<sub>19</sub> and Figure 6(ii) is a representation of the 3D structure of suramin;

Figures 7A-D are graphs of erythrocyte invasion by merozoites (as a percentage of control samples) in the presence of different concentrations of various suramin analogues; and

Figure 8 is a sequence alignment of the amino acid residue sequence of MSP1<sub>42</sub> proteins from various Plasmodium species.

A very large number of suramin analogues are known and, for many of these, toxicity data are already available, so it would be relatively straightforward for those skilled in the art, with the benefit of the present disclosure, to identify low-toxicity analogues with suitable inhibitory properties for MSP1<sub>42</sub> processing and/or merozoite invasion of erythrocytes.

### Examples

The inventors carried out experiments to investigate what effect, if any, suramin or a suramin analogue might have on invasion of erythrocytes by merozoites.

#### Example 1 – methods

Suramin, sodium salt, (Antrypol (ICI)) and suramin analogues (synthesised and described by Balaban & King in 1927, [J. Chem. Soc. 3068-3097]) (see Table 1) were provided by Mr Terry Scott-Finnigan (Division of Parasitology, National Institute for Medical Research, London, UK and Dr Roy Bicknell, IMM, Oxford). Data on their maximum tolerated doses (MTD) in mice is detailed by Braddock *et al*, (1994 Br. J. Cancer 69, 890-898). Naphthalene-1,3,6-trisulphonic acid (NTS) tri-sodium hydrated salt was purchased from Fluka (#70310).

#### *In vitro culture and synchronisation of P. falciparum*

Asexual blood stages of *P. falciparum* (FCB-1) were maintained at 37°C in RPMI 1640/Albumax medium (Gibco) supplemented with 2mM L-glutamine as previously

described (Blackman, 1994 Methods Cell Biol. 45, 213-220). Cultures were gassed with 7% CO<sub>2</sub>, 5% O<sub>2</sub> and 88% N<sub>2</sub> and maintained by routine passage in fresh human erythrocytes. Parasites were synchronised by Percoll and sorbitol treatment (Holder & Freeman, 1982 J. Exp. Med. 156, 1528-1538); schizonts were purified by centrifugation over Percoll and then returned to culture in the presence of fresh erythrocytes. After 4 h, during which time released merozoites invaded erythrocytes, the cells were treated with 5% sorbitol for 10 min to lyse the residual schizonts, before returning the parasites to culture.

*P. falciparum in vitro invasion and growth inhibition assays*

Compounds were tested for their ability to inhibit invasion *in vitro*, using two approaches: a short-term assay in which the number of newly invaded erythrocytes was counted using microscopy, and a growth assay measuring uptake of [<sup>3</sup>H] hypoxanthine. In the short term-assay (described previously by Blackman *et al*, 1990 J. Exp. Med. 172, 379-382), compounds were incubated with purified *P. falciparum* schizonts at ~2% parasitaemia and ~2% haematocrit in triplicate cultures. After between 6 and 24 h, blood smears were stained with Giemsa's reagent and examined by microscopy. The number of newly invaded ring stages was counted and inhibition of invasion was expressed as percent invasion relative to an untreated culture  $[(I_C / (I_C + U_C)) / (I_A / (I_A + U_A)) \times 100\%]$ , where  $I_C$  is the number of erythrocytes infected with ring stages,  $U_C$  is the number of uninfected erythrocytes in the presence of the compound,  $I_A$  is the number of erythrocytes infected with ring stages, and  $U_A$  is the number of uninfected erythrocytes in the absence of the compound.

Suramin and NTS were also assayed for *P. falciparum* growth inhibition using [<sup>3</sup>H] hypoxanthine uptake (generally as described by Desjardins *et al*, 1979 Antimicrob. Agents Chemother. 16, 710-718). Serial dilutions of the compounds in 96-well plates were incubated at 37°C with purified mature schizonts at a final parasitaemia of ~0.5% and a haematocrit of ~2% in triplicate cultures. Following incubation for about 24 h, [<sup>3</sup>H] hypoxanthine (0.5 µCi/ well) was added for a further 18 h, and the cells were harvested onto glass fibre filters (Filtermat A, Wallac, Turku, Finland) using a cell harvester. The

filters were wetted with scintillation cocktail and the bound radioactivity counted in a  $\beta$ -counter. Control incubations without compound or without parasitised erythrocytes were included in each experiment. The amount of radioactivity in each sample was expressed as a percentage of activity in the control wells containing no compound. Three independent experiments were performed for each compound.

### Example 1 – results

Microscopic examination of parasite cultures following staining with Giemsa's reagent suggested that suramin inhibited merozoite invasion of erythrocytes. Free merozoites were observed in the stained samples, implying that suramin did not inhibit merozoite release from schizonts. These finds were confirmed by the more quantitative [ $^3\text{H}$ ] hypoxanthine incorporation assay. The results (in triplicate) of the hypoxanthine assay are shown in Figure 2. The open symbols show the results for suramin, the closed symbols are the results for NTS. The amount of radioactivity incorporated is proportional to the number of growing parasites and the effect of the compound is expressed relative to incorporation in the absence of compound (% relative parasite growth).

Figure 2 shows that suramin inhibited erythrocyte invasion in a dose dependent manner with an  $\text{IC}_{50}$  of  $60 \mu\text{M} \pm 9$ , whereas NTS did not inhibit invasion even at  $200 \mu\text{M}$  (the highest concentration tested).

### Example 2

In order to investigate the basis for the inhibition of invasion noted above, the inventors explored the effect of suramin and NTS on secondary processing of MSP1.

#### *P. falciparum* MSP1 secondary processing assay

Merozoites were purified as described previously (Blackman 1994 Methods Cell Biol. 45, 213-220) after release from mature schizonts into growth medium supplemented with 2 mM EGTA. Merozoites were harvested by centrifugation, followed by sequential passage through pre-wetted filters of 3 and  $1.2 \mu\text{m}$  pore size. Merozoites were washed in  $\text{Ca}^{2+}$ - and  $\text{Mg}^{2+}$ -free PBS in the presence of protease inhibitors (leupeptin, antipain and aprotinin



at  $10 \mu\text{g ml}^{-1}$ ). The merozoites were then washed and resuspended in 50 mM Tris-HCl pH 7.2, 5 mM  $\text{CaCl}_2$ , 1 mM  $\text{MgCl}_2$ , plus leupeptin, antipain and aprotinin at  $10 \mu\text{g ml}^{-1}$  (processing buffer), and divided into 18  $\mu\text{l}$  aliquots. Two microlitres of either diluted compound or reaction buffer were added and the samples incubated for 1 h at  $37^\circ\text{C}$ . Control assays included those in which processing was prevented by immediate addition of either SDS, 1 mM PMSF or 5 mM EGTA; and those in the absence of compound or in the presence of 0.2 mM TLCK (tosyl-L-lysyl chloromethyl ketone). After 1 h the reaction was stopped, and MSP1 processing was analysed using a Western blot-based assay (Blackman 1994 Methods Cell Biol. 45, 213-220). The blots were probed with a rabbit anti-MSP1<sub>33</sub>/MSP1<sub>42</sub> antibody and the bands corresponding to MSP1<sub>42</sub> and MSP1<sub>33</sub> polypeptides were visualised by enhanced chemiluminescence.

Purified merozoites were incubated in the presence or absence of suramin or NTS and polypeptides subjected to SDS-PAGE and Western blotting. The results are shown in Figures 3A and 3B.

Figure 3A is a picture of blot of *P. falciparum* comprising: lane 1 – SDS; lane 2 – no further addition; lane 3 – 1mM PMSF (potent protease inhibitor); lane 4 – 200  $\mu\text{M}$  NTS; or lane 5 – 200  $\mu\text{M}$  suramin. The major band is that corresponding to MSP1<sub>42</sub>; the minor, lower band (where present) is MSP1<sub>33</sub>, one of the products of secondary processing of MSP1<sub>42</sub>. The absence of MSP1<sub>33</sub> (as in lanes 1, 3 and 5) is indicative of inhibition of MSP1<sub>42</sub> secondary processing.

Further experiments (results shown in Figure 3B) demonstrated that the observed inhibition of MSP1<sub>42</sub> processing by suramin was dose-dependent. Figure 3B shows the results of a western blot, using the assay protocol described above, with *P. falciparum* merozoites incubated in: lane 1 – SDS; lane 2 – no further addition; lane 3 – 1mM PMSF; lane 4 – 200  $\mu\text{M}$  suramin; lane 6 – 50  $\mu\text{M}$  suramin; lane 7 – 12.5  $\mu\text{M}$  suramin; lane 8 – 3.1  $\mu\text{M}$  suramin and lane 9 – 1.25  $\mu\text{M}$  suramin.

### Example 3 – Fluorimetry Assay and Isothermal Titration Calorimetry

The nature of the interaction between suramin and MSP1<sub>42</sub> was investigated further. A number of MSP1-based recombinant proteins were prepared from *P. falciparum* and *P. vivax* as described below.

#### *P. falciparum* MSP-1<sub>42</sub>

The pETATPf MSP1<sub>42</sub> plasmid (described by Angov *et al*, 2003 Mol. Biochem. Parasitol. 128, 195-204) was used to express a His<sub>6</sub>-tagged MSP1<sub>42</sub> protein (*P. falciparum* 3D7 clone, Accession Number Z35327). The DNA was used to transform *E. coli* BL21 (DE3) cells and then clones were selected on plates of agar containing ampicillin. Cells expressing the modified MSP1<sub>42</sub> were grown to mid log phase (OD<sub>600</sub> 0.5-0.8) at 37°C, then after the incubation temperature had been reduced to 25°C, the protein expression was induced by addition of 0.1 mM IPTG. After 2 h of further growth the bacterial cells were harvested by centrifugation. The resulting cell paste was resuspended in lysis buffer (10 mM sodium phosphate, 50 mM NaCl, 10 mM imidazole, pH 6.2) and lysed by microfluidization. The final lysate was then adjusted to 500 mM NaCl and 1% (v/v) Tween 80 (final concentrations) and incubated for a further 20 min with mixing. After centrifugation at 30,000 x g for 1 h the supernatant was applied to a column containing Ni<sup>2+</sup> NTA Superflow resin (Qiagen). The column was washed extensively and sequentially with the following buffers: 10 mM sodium phosphate, 500 mM NaCl, 10 mM imidazole, 0.5% Tween 80, pH 6.2; 10 mM sodium phosphate, 75 mM NaCl, 20 mM imidazole, pH 8.0. Bound protein was eluted with 10 mM sodium phosphate, 75 mM NaCl, 160 mM imidazole, pH 8.0.

#### *P. falciparum* MSP1<sub>19</sub>

*Plasmodium falciparum* MSP1<sub>19</sub> (Swiss-Prot 04933) was expressed from a synthetic gene optimised for *Pichia pastoris* expression (European Patent No. EP1180120). This construct, inserted at the SnaBI site of vector pPIC9K (Invitrogen), contained the N-terminal leader sequence YHHHHHHIEGRH preceding the MSP1<sub>19</sub> sequence. A point mutation (Ser3 to Ala) was also introduced to eliminate N-glycosylation at Asn1. Following purification of MSP1<sub>19</sub> as described previously (Morgan *et al*, 1999 J. Mol.

Biol. 289, 113-122), the N-terminal tag was cleaved with Factor Xa (New England Biolabs) and MSP1<sub>19</sub> was purified by gel filtration (Superdex 200). The final product contained the 96 amino acid MSP1<sub>19</sub> fragment, preceded by a single His residue.

The MSP1<sub>42</sub> protein from *P. vivax* was cloned into plasmid jmp28, a modified pET-28 vector that encodes an N-terminal peptide MHHHHHHHIEGRWIL immediately upstream of the inserted sequence. *P. vivax* (Belem strain) DNA was used as a template for PCR based cloning. The sequence of the expressed protein (following the vector encoded N-terminal peptide) corresponded to residues Asp 1325 to Ser 1704, Accession number A39401.

The protein was expressed in BL21(DE3)pLysS cells by induction of a culture at an OD<sub>600</sub> of 0.6-0.8 with IPTG at a final concentration of 1 mM. The cells were induced for 3 h at 37°C and then harvested by centrifugation. The cells were lysed using the Bugbuster protein extraction reagent (Novagen), after centrifugation of the cell lysate, the pellet containing the MSP1<sub>42</sub> was dissolved in 6M Guanidine-HCl, 100 mM Na<sub>2</sub>HPO<sub>4</sub>, 10 mM Tris-HCl, pH 8.0. This was applied to a Ni-NTA column, washed with 10 column volumes of the loading buffer, followed by 6 column volumes of 8 M Urea, 100 mM Na<sub>2</sub>HPO<sub>4</sub>, 10 mM Tris-HCl, at pH 7.0, and 6 column volumes at pH 6.1. The protein was eluted in 3 column volumes 8 M Urea, 100 mM Na<sub>2</sub>HPO<sub>4</sub>, 10 mM Tris-HCl, pH 4.3.

The protein was refolded by rapid dilution (1:50) into refolding buffer (55 mM Tris-HCl, pH 8.2, 264 mM NaCl, 11 mM KCl, 550 mM GuHCl, 1.1 mM EDTA, 1 mM GSH, 0.1 mM GSSG) and incubation overnight at 18°C. The protein was concentrated, then purified by gel filtration on a Superdex 200 (26/600 mm) column (Amersham) equilibrated in 20 mM Tris-HCl, 250 mM NaCl, pH 8.0. Protein containing peaks were pooled, diluted 5-fold into 20 mM Tris-HCl, pH 8.0 and applied to a Mono-Q HR 5/5 column (Amersham) and eluted using a NaCl gradient from 0-250 mM. The protein eluted at 150-250 mM NaCl. The fractions containing MSP1<sub>42</sub> were pooled and dialysed extensively with PBS.

*P. vivax* MSP1<sub>33</sub> was expressed using the *P. vivax* MSP1<sub>42</sub> clone, above, as a template for PCR, followed by ligation of the product into vector pET30Xa/LIC. The sequence runs from Asp 1325 to Ser 1618 (Accession number A39401) following the N-terminal purification tag. The vector was used to transform BL21(DE3)pLysS cells. For expression, a culture of the transformed cells was induced at an OD<sub>600</sub> of 0.6-0.8 at 37°C with 1mM IPTG for 3 h. The cells were harvested by centrifugation and the cell pellet lysed using the Bugbuster protein extraction reagent (Novagen). The cell lysate was cleared by centrifugation and the supernatant loaded directly onto a Ni-NTA column. The column was washed with 10 volumes of 50 mM NaH<sub>2</sub>PO<sub>4</sub>, 300 mM NaCl, 10 mM imidazole, pH 8.0, then with 6 column volumes of the same buffer containing 30 mM imidazole, and finally eluted with 3 column volumes of the buffer with 250 mM imidazole. The eluted protein was then loaded directly onto a Superdex 200 (26/600) column (Amersham) equilibrated in 20 mM Tris-HCl, 250 mM NaCl, pH 8.0 and purified by gel filtration. The fractions containing MSP-1<sub>33</sub> were pooled and dialysed extensively with PBS.

#### *Binding of suramin to MSP1 assayed by fluorimetry*

The intrinsic fluorescence of suramin was used to determine its capacity to bind to the various MSP1-derived proteins. The fluorescence was measured using a Perkin Elmer LS-3B or a Spex Fluoro Max 2 fluorimeter. Suramin was excited at a wavelength of 315 or 330 nm with 2.5 nm resolution, the emission spectra were measured at wavelengths ranging from 350-450 nm. Suramin and the MSP1 proteins were diluted in 20 mM NaH<sub>2</sub>PO<sub>4</sub>, 150 mM NaCl at pH 7.2 and analysed at 10 or 20°C. Titrations were performed by adding aliquots of the suramin solution to the MSP1 solution. In controls, suramin was titrated into buffer. The K<sub>d</sub> for the suramin-MSP1 binding was determined from three independent experiments.

Typical results are shown in Figure 4. The initial solution contained 0.3 μM MSP1<sub>42</sub>, 20mM NaH<sub>2</sub>PO<sub>4</sub>, 0.15M NaCl pH 7.2 at 20°C to which μl aliquots of 100μM suramin were added.

The intrinsic fluorescence of suramin is low when excited at 315 nm, but when it binds to MSP1<sub>42</sub> it shows a pronounced increase in emission intensity with the maximum emission slightly shifting from 408 to 411 nm (Fig. 4 insert). This fluorescence enhancement was used to measure binding of suramin to MSP1<sub>42</sub> from *P. falciparum*. Following titration of a solution of MSP1<sub>42</sub> with suramin, the marked increase in the fluorescence intensity was measured as a function of suramin concentration (Fig. 4). Analysis of the binding data revealed that suramin binds to MSP1<sub>42</sub> from *P. falciparum* with a  $K_d$  of  $0.22 \mu\text{M} \pm 0.04$ .

In addition to the fluorimetry, isothermal titration calorimetry (ITC) was performed using a Microcal omega VP-ITC (MicroCal Inc., Northampton, MA). The proteins were dialyzed extensively against the ITC buffer (phosphate buffered saline, pH 7.4). All experiments were performed at 25°C. The cell, 1.425 ml, contained 30  $\mu\text{M}$  MSP1<sub>42</sub> or MSP1<sub>33</sub> and these were titrated by injection of a total of 290  $\mu\text{l}$  of 600  $\mu\text{M}$  suramin. The heat of dilution of suramin into buffer was determined in control experiments. The data were fitted by least-squares methods using the evaluation software, Microcal Origin version 5.0 provided by the manufacturer. Each experiment was performed twice.

MSP1<sub>42</sub> and MSP1<sub>33</sub> from *P. vivax* were prepared as described above and their suramin binding properties analysed by isothermal titration calorimetry. MSP1<sub>33</sub> from *P. falciparum* was soluble to only 0.15 mg/mL in aqueous buffers and aggregated significantly even at these concentrations, preventing measurements of suramin binding *in vitro*. However MSP1<sub>33</sub> from *P. vivax* was soluble to at least 20 mg/mL and hence amenable to *in vitro* methods of measuring suramin binding. *P. vivax* MSP1<sub>42</sub> was found to bind suramin with a similar  $K_d$  ( $0.3 \pm 0.1 \mu\text{M}$ ) to that measured for *P. falciparum* MSP1<sub>42</sub>. MSP1<sub>33</sub> from *P. vivax* showed a high affinity site that was 5 fold weaker than for MSP1<sub>42</sub> ( $1.5 \pm 0.5 \mu\text{M}$ ). The  $\Delta H$  values ( $11 \pm 1$  kcal/mol for MSP1<sub>42</sub> and  $12 \pm 1$  kcal/mol for MSP1<sub>33</sub>) are also very similar. At the high concentrations ( $>30 \mu\text{M}$ ) required for ITC analysis there was evidence for some non-specific binding. When analysed by fluorimetry *P. vivax* MSP1<sub>42</sub> and MSP1<sub>33</sub> showed large binding-induced enhancements of the intrinsic suramin fluorescence similar to that observed with *P. falciparum* MSP1<sub>42</sub>. Thus *P. falciparum* MSP1<sub>42</sub>, *P. vivax* MSP1<sub>42</sub> and *P. vivax* MSP1<sub>33</sub>

all exhibited large enhancements of suramin fluorescence on binding. The suramin binding for MSP1<sub>33</sub> is only fivefold weaker than that for MSP1<sub>42</sub> and this taken together with the similar large induced fluorescence enhancements indicates that there is a similar hydrophobic suramin binding pocket in the two proteins.

#### Example 4 – NMR Studies with MSP1<sub>19</sub>

No fluorescence enhancement was observed when suramin was added to MSP1<sub>19</sub> from *P. falciparum* and thus suramin binding to MSP1<sub>19</sub> could not be determined by fluorescence measurements. However, using NMR it was possible to follow the changes in chemical shifts of the <sup>1</sup>H and <sup>15</sup>N signals in <sup>1</sup>H-<sup>15</sup>N HSQC experiments when titrating <sup>15</sup>N labelled MSP1<sub>19</sub> with suramin.

NMR experiments were carried out on Varian spectrometers operating at proton frequencies of 500, 600 and 800 MHz. Suramin (0.03 – 16.0 mM) <sup>1</sup>H spectra were recorded at 5 to 35°C. The assignments of the <sup>1</sup>H signals of suramin (except for the NH signals) were made by analysis of the 2D gradient selected double quantum filtered COSY spectrum at 500 MHz, 25°C on a sample containing 1 mM suramin in 50 mM potassium phosphate, 100 KCl, 90%/H<sub>2</sub>O/10%D<sub>2</sub>O at pH 6.5). The NH assignments were made on the basis of NOESY (nuclear Overhauser effect spectroscopy) experiments. MSP1<sub>19</sub>/suramin samples were examined with either unlabelled or <sup>15</sup>N labelled MSP1<sub>19</sub> in 50 mM sodium phosphate and 100 mM NaCl in 90% H<sub>2</sub>O/10% D<sub>2</sub>O at pH 6.5 (pH values are pH meter readings uncorrected for deuterium isotope effects). A titration was carried out by mixing two samples each containing 0.1 mM MSP1<sub>19</sub> and with one also containing 16 mM suramin. 1D <sup>1</sup>H and 2D <sup>1</sup>H-<sup>15</sup>N HSQC NMR spectra were recorded for each concentration of suramin. NOESY spectra were recorded on MSP1<sub>19</sub>/suramin samples in D<sub>2</sub>O (1.76 mM protein and 6 mM suramin in 50 mM sodium phosphate and 100 mM NaCl at pH 6.5); these spectra were compared to MSP1<sub>19</sub> spectra recorded in the absence of suramin to detect any suramin induced changes in <sup>1</sup>H chemical shifts for protein side chain resonances.

At the maximum concentration of suramin used, 16 mM, about 50% of MSP1<sub>19</sub> was complexed with the ligand. The residues that showed the largest shifts on addition of suramin were Ile 2, His 5, Phe 19, His 21, Leu 22 and Arg 25. The shifts were fitted by non-linear regression analysis to a single binding curve and gave an average  $K_d \sim 15 \text{ mM} \pm 5$ .

Fig. 5A shows binding curves illustrating the suramin concentration dependence of the <sup>1</sup>H chemical shifts of the NH signals from His 5 and Leu 22 residues of MSP1<sub>19</sub>.

Figure 6(i) is a representation of the structure of MSP1<sub>19</sub> as determined by nmr (Morgan *et al.*, 1999 J. Mol. Biol. 289, 113-122). The residues affected by suramin binding are shown in black. N and C indicate the sites of the protein termini.

Figure 6(ii) shows suramin in its extended conformation, to the same scale, for comparison.

### Example 5 – Suramin analogues

#### *Suramin-analogues inhibit erythrocyte invasion and MSP1 secondary processing*

A number of suramin analogues were examined in order to probe the features of the molecule necessary for binding to MSP1 and for inhibiting *P. falciparum* MSP1 processing. Four series of symmetrical compounds (Groups A-D, Table 1) differing in the number of central aminobenzoyl urea units, and having various substitutions on the terminal naphthyl rings, were examined.

The results of invasion assays are shown in Figures 7A-D, which are graphs of erythrocyte invasion (as a percentage of control experiments in the absence of analogue) against concentration of analogue (μM). The error bars denote the standard deviation.

None of the Group A compounds or the single group D compound (D1) inhibited invasion *in vitro* at 200 μM, the highest concentration tested (Fig. 7A and D). However, Group B

and C compounds inhibited invasion *in vitro* (Fig. 7B and C), with  $IC_{50}$  values similar to that of suramin. The suramin analogues differ in toxicity. The least toxic inhibitory compound, B1, is 10 times less toxic than suramin. The various suramin analogues were also examined for their ability to inhibit MSP1<sub>42</sub> processing, as described above. These assay results (omitted for brevity) demonstrated that the compounds that inhibit invasion also inhibit MSP1<sub>42</sub> processing, whereas those that do not inhibit invasion also do not inhibit processing, when tested at the concentration of 200  $\mu$ M. Similar parasite invasion and MSP1 processing assays could not be carried out for *P. vivax* since this species cannot be cultured *in vitro*.

#### Example 6

This example relates to an illustration of an assay suitable for performing the screening method of the invention.

In the example, the comparison is suramin, but many other compounds (especially analogues of suramin of groups B and C in Table 1) could be employed. Suramin is especially convenient as it possesses intrinsic fluorescence which, upon binding to MSP1<sub>42</sub> or a fragment thereof, is greatly enhanced and this enhanced fluorescence can be used as a measure of MSP1<sub>42</sub>-binding.

The assay is performed in a conventional microtitre plate. The wells of the plate comprise purified MSP1<sub>42</sub>, derived from *P. falciparum* or *P. vivax* at an appropriate concentration (e.g. about 1  $\mu$ M). To respective individual wells (perhaps in duplicate or triplicate) the test substances would be added, typically at a concentration of about 5-500  $\mu$ M preferably about 10-50  $\mu$ M. At concentrations above about 500  $\mu$ M a large number of compounds might exhibit non-specific binding, which is not of interest from a clinical viewpoint. If desired the test substances may be tested at a number of different concentrations. A fluorescence measurement is then performed, with excitation at about 330nm and detecting emission at about 440nm. (With suramin the precise wavelengths used are not very important, as the excitation/emission spectra are very broad: thus, excitation could be



performed at any wavelength in the range 305-375nm and emission could be detected over the range 380-450nm).


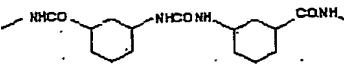
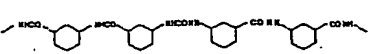
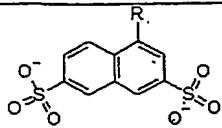
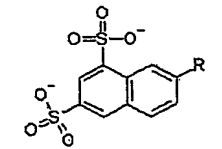
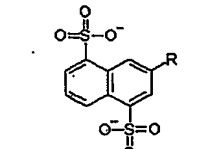
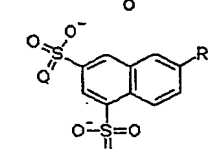
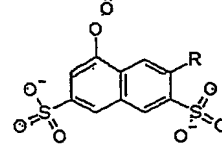
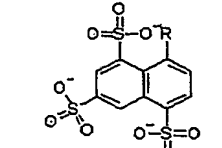
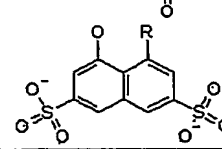
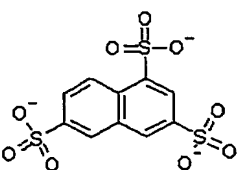
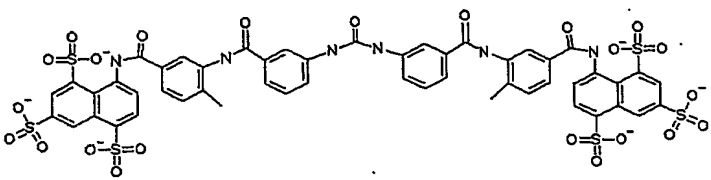
This initial fluorescence measurement will detect those test substances which are themselves naturally fluorescent at a relevant wavelength (or become so upon binding to MSP1<sub>42</sub> or a fragment thereof), which can then be allowed for when interpreting the assay results.

Suramin, the comparison substance, is then added to the wells at a suitable concentration, which will typically be less than the concentration of the test substance. Typically the concentration of suramin will be in the range 0.5µM-10µM. The microtitre plate is then left to incubate a suitable period of time at a suitable temperature (e.g. 1hr at 20°C, or say 30 minutes at 37°C) to allow any reaction to proceed. A second fluorescence measurement is then made (typically using the same excitation/emission wavelengths as for the first measurement). Those wells in which the suramin-mediated fluorescence is abolished or significantly reduced indicates that the test substance has bound to MSP1<sub>42</sub> (or the fragment thereof) at the same site as would have been occupied by suramin and has therefore prevented suramin binding.

Test substances of potential interest identified in this way may then be made the subject of further analysis and investigation, for example by assay in the MSP1 secondary processing and/or inhibition of invasion tests as described herein.

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TABLE 7

Naphthylamine derivative	Urea derivative (R)		
	(A) 	(B) <i>m</i> -aminobenzoyl 	(C) <i>m'</i> -aminobenzoyl- <i>m</i> -aminobenzoyl 
	A1 (CPD 6)	B1	C1
	A2 (CPD 1)	B2 (CPD 9)	C2 (CPD 10)
	A3 (CPD 3)*	B3	C3 (CPD 11)
	A4 (CPD 4)	B4 (CPD 12)	C4 (CPD 13)
	A5 (CPD 7)	B5 (CPD 14)	C5 (CPD 15)
	A6 (CPD 2)		C6 (CPD 16)*
			C7 (CPD 8)
(D) Carbonyl derivative D1 (CPD 5)	Naphthalene trisulphonate 	Suramin 	

\* not tested

Claims

1. A method of screening a test substance for possession of binding activity for MSP1<sub>42</sub> or a fragment thereof, the method comprising the steps of: combining or contacting, in any order,
  - (i) a molecule comprising MSP1<sub>42</sub> or a fragment thereof,
  - (ii) the test substance, and
  - (iii) a comparison substance known to have binding activity for MSP1<sub>42</sub> or a fragment thereof;and determining the presence and/or amount, if any, of comparison substance and/or test substance bound to the MSP1<sub>42</sub> or fragment thereof.
2. A method according to claim 1, wherein the comparison substance inhibits processing of MSP1<sub>42</sub> and/or inhibits merozoite invasion of erythrocytes.
3. A method according to claim 1 or 2, wherein the comparison substance is suramin or a suramin analogue.
4. A method according to any one of the preceding claims wherein the comparison substance and/or the test substance is labelled to facilitate detection.
5. A method according to any one of the preceding claims wherein the fragment of MSP1<sub>42</sub> is MSP1<sub>19</sub> or MSP1<sub>33</sub>.
6. A method according to any one of the preceding claims wherein binding of the comparison and/or test substance is determined by fluorescence measurements.
7. A method according to any one of the preceding claims, wherein the test substance is screened against a number of different MSP1<sub>42</sub> molecules or fragments thereof.

8. A method according to any one of the preceding claims, wherein the MSP1<sub>42</sub> molecule or fragment thereof is a mutant of a naturally-occurring wild type sequence.
9. Use of suramin or an analogue thereof in the preparation of a medicament to treat or prevent malarial disease in a mammalian subject.
10. A pharmaceutical composition comprising suramin or an analogue thereof for use in the prevention and/or treatment of malarial disease in a mammalian subject.
11. A pharmaceutical composition according to claim 10, wherein the active ingredient is identified by performance of a method in accordance with any one of claims 1-8.
12. A method substantially as hereinbefore described and with reference to the accompanying drawings.

ABSTRACT

Title:     Screening Assay

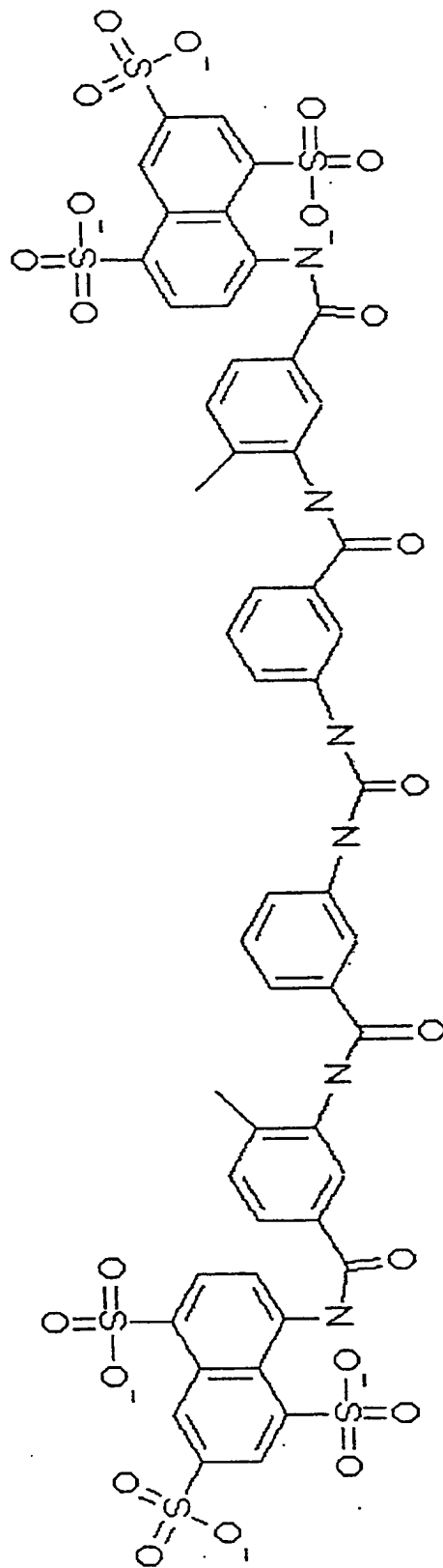
Disclosed is a method of screening a test substance for possession of binding activity for possession of binding activity for MSP<sub>42</sub> or a fragment thereof, the method comprising the steps of: combining or contacting, in any order,

- (i) a molecule comprising MSP<sub>142</sub> or a fragment thereof,
- (ii) the test substance, and
- (ii) a comparison substance known to have binding activity for MSP<sub>142</sub> or a fragment thereof;

and determining the presence and/or amount, if any, of comparison substance and/or test substance bound to the MSP<sub>142</sub> or fragment thereof.

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Fig. 1



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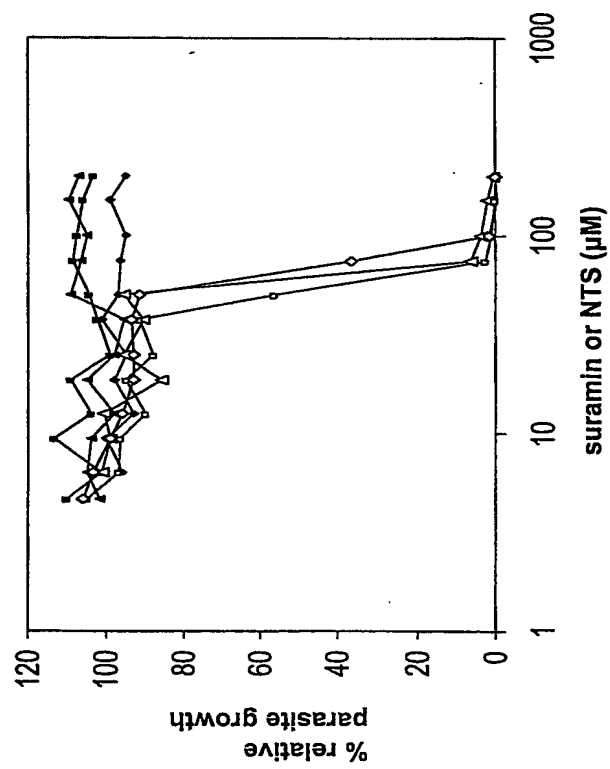
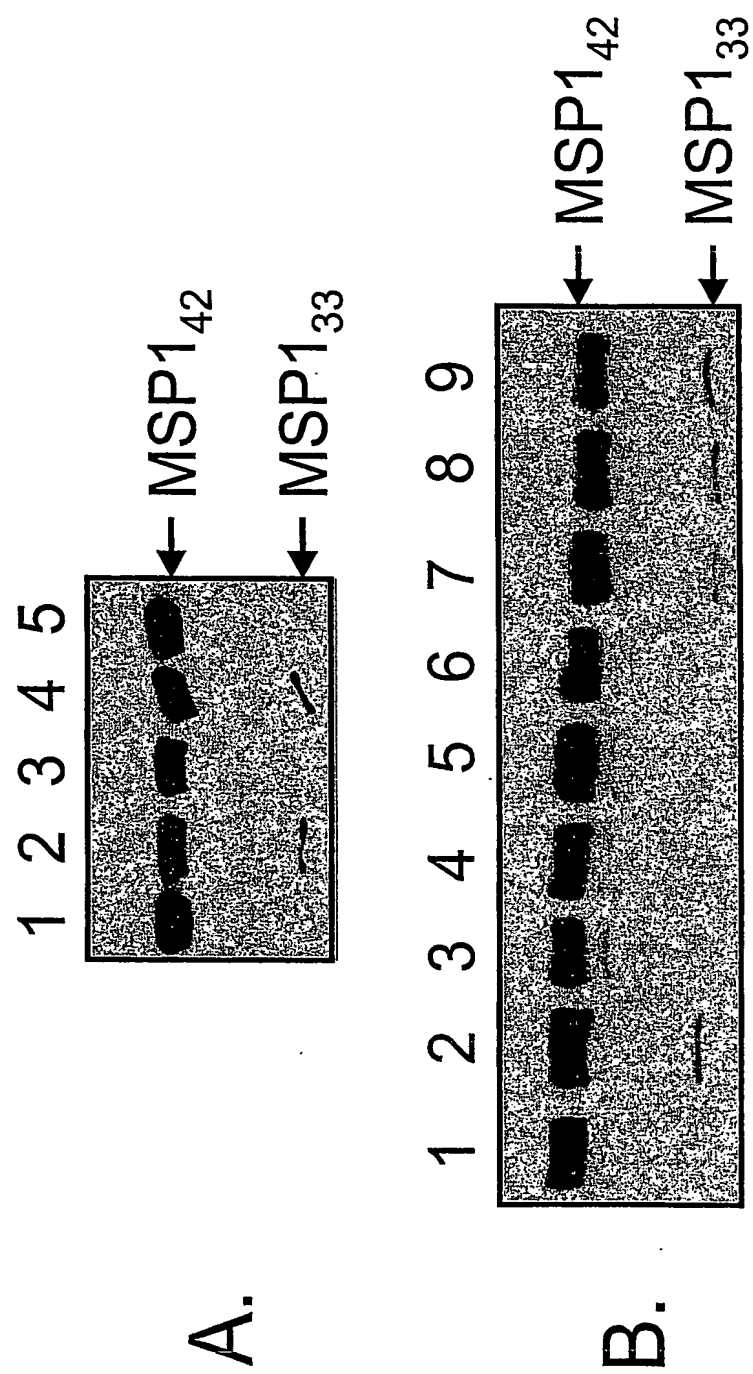


Fig. 2

Fig. 3





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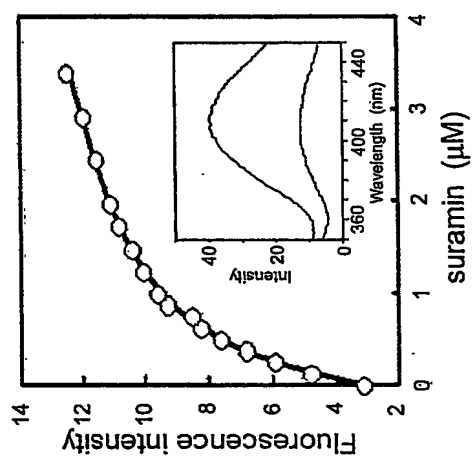


Fig. 4

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Fig. 5A

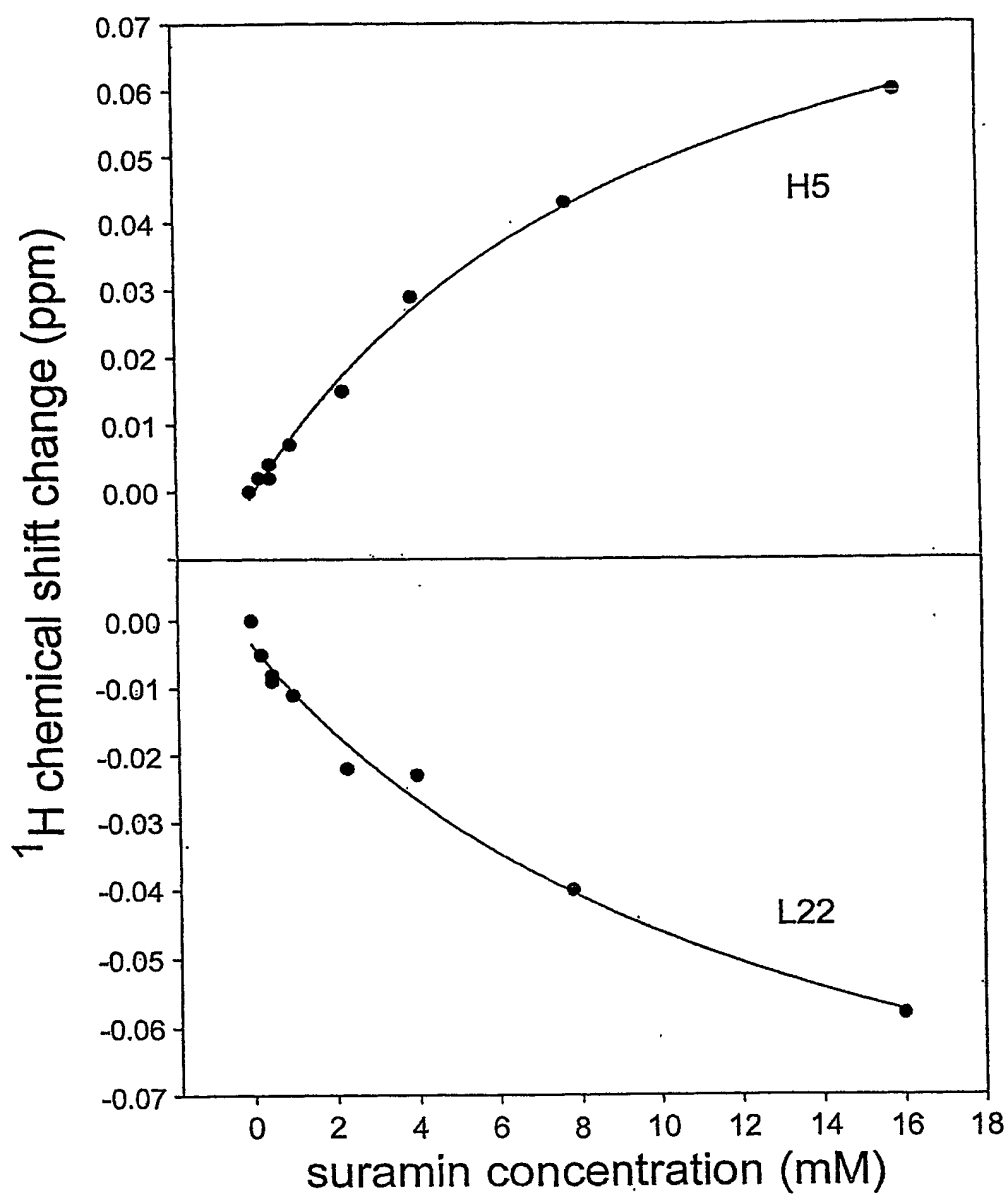


Fig. 5B

Fig. 6 (ii)

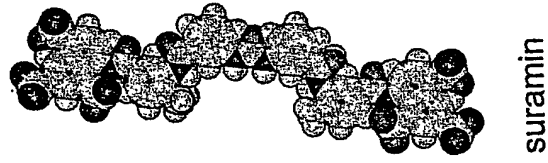
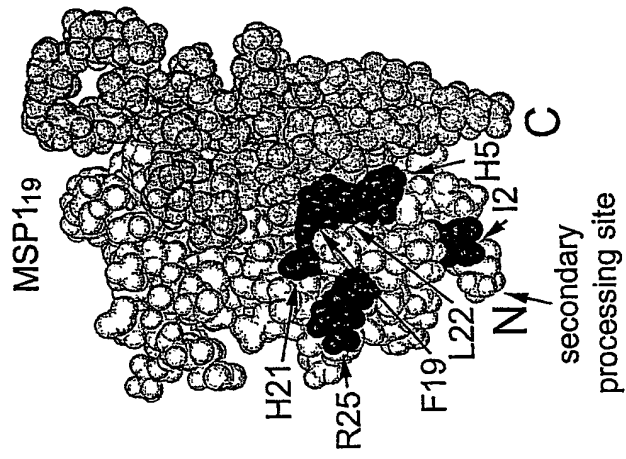
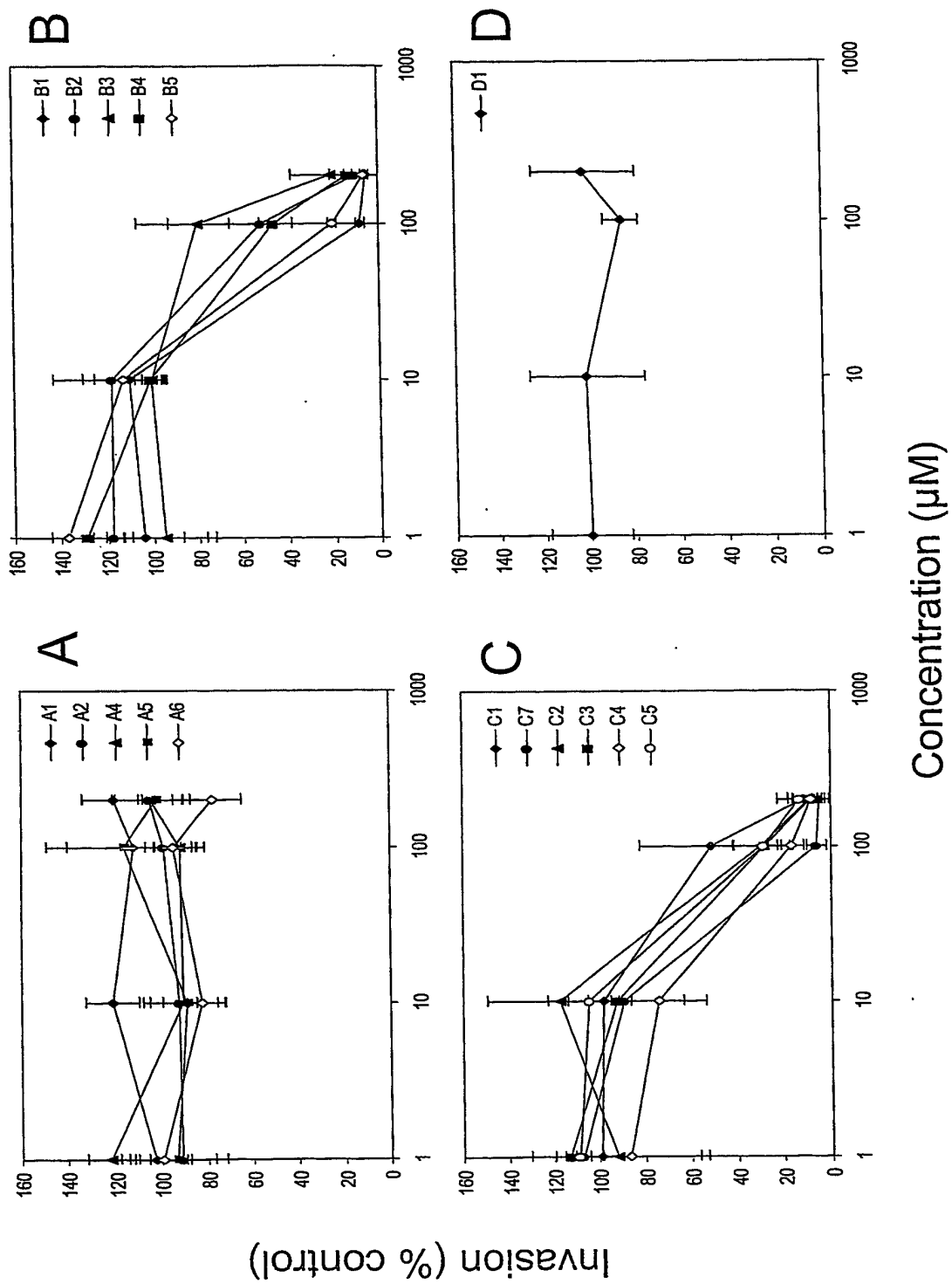


Fig. 6 (i)



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Fig. 7



10 20 30 40 50 60

Pfwel42	GEAVT	PSVID	NILSKIENEY	EVLYLKPLAC	VYRSLKKOLE	NNVMHFNVNV	KDILNSRFNK
PfMAD42	GEAIS	VTMD	NILSGFENEY	DVIYLKPLAC	VYRSLKKOLE	KNITFTFNLNL	NDILNSRLKK
CAMP-42	GEAIS	VTMD	NILSGFENEY	DVIYLKPLAC	VYRSLKKOLE	KNITFTFNLNL	NDILNSRLKK
FC27-42	GEAIS	VTMD	NILSGFENEY	DVIYLKPLAC	VYRSLKKOLE	KNITFTFNLNL	NDILNSRLKK
3D7-42	GEAIS	VTMD	NILSGFENEY	DVIYLKPLAC	VYRSLKKOLE	KNITFTFNLNL	NDILNSRLKK
Pv142	GEAES	EAPE	ILVPAGISDY	DVYLYKPLAC	MYKTLKKOLE	NEVNAFNTNI	TDMLDSRLKK
Pv242	GEAES	EAPE	ILVPAGISDY	DVYLYKPLAC	MYKTLKKOLE	NEVNAFNTNI	TDMLDSRLKK
Pcyn42	GEAES	EAPE	ILVPQGINNEY	DVYLYKPLAC	MYKTLKKPLE	NEVNALNTNI	IDMLDSRLKK
Pk42					-----KKOLE	NEVAAFNTNI	TDMLDSRLKK
Py42	EQSED	APEK	DILSEETNES	LYVYTKREGS	TYKSLKKHML	REFSTIKEDM	TNGLNNKSQK
Pb42	EQSEN	AQEK	NILEAFKNES	EYLYARS LGI	TYKSLKKHMI	REFSTIKEDM	TTGLNNKLEK
Pc42	AQSTD	EEVK	DILDAFKSEN	EYLYTKSLGN	TYKSFKKHML	KEFSMIKEDI	MTGLNLYKLEK

70 80 90 100 110 120

Pfwel42	RENEKVNLES	DLIPYKDLTS	SNYVVKDEYK	FLNKEKRDKE	LSSYNYIKDS	IDTDINFAND
PfMAD42	RKYFLDVLES	DLMOQFKISS	NEYIIEDSFK	LLNSEQKNTL	LKSYKYIKES	VENDIKFAQE
CAMP-42	RKYFLDVLES	DLMOQFKISS	NEYIIEDSFK	LLNSEQKNTL	LKSYKYIKES	VENDIKFAQE
UPA-p42	RKYFLDVLES	DLMOQFKISS	NEYIIEDSFK	LLNSEQKNTL	LKSYKYIKES	VENDIKFAQE
FC27-42	RKYFLDVLES	DLMOQFKISS	NEYIIEDSFK	LLNSEQKNTL	LKSYKYIKES	VENDIKFAQE
3D7-42	RKYFLDVLES	DLMOQFKISS	NEYIIEDSFK	LLNSEQKNTL	LKSYKYIKES	VENDIKFAQE
Pv142	RNYFLEVLNS	DLNPEKYS	GEYIIEKDEYK	LLDLEKKKKL	LGSYKYIGAS	IDKDLGTAND
Pv242	RNYFLEVLNS	DLNPEKYS	GEYIIEKDEYK	LLDLEKKKKL	LGSYKYIGAS	IDMDLATAND
Pcyn42	RNYFLDVINS	DLNPFKSI	GEYIIEKDEYK	LLDLEKKKKL	LGSYKYIGAS	VDKDMVTAND
Pk42	RNYFLDALDS	ELNPEKYS	GEYIIEKDEYK	LLDLEKKKKL	LGSYKYIGAS	VDKDLITAKD
Py42	RNDFLEVLNS	ELDLFKDLST	NKYVIRNEYQ	LLDNDKKKDKQ	IVNLKYATKG	INEDIETTTD
Pb42	RNDFLEVLNS	ELDLFKDLST	NKYVIRNEYQ	LLDNDKKKDKQ	IVNLKYAAGK	VNEDIETTTD
Pc42	RNDFLDVLSY	ELALEKDINT	NKFVVKNEYQ	LLDNDKKKDKQ	MINLKYAIGK	VTEDIETTTD

130 140 150 160 170 180

Pfwel42	VIGYKILSE	KYKSDLDSIK	KYIN-----			
PfMAD42	GISYIEKVL	KYKDDLESIK	KVIKEEKEKF	P-----	SSPPT	TPPSPAKT--
CAMP-42	GISYIEKVL	KYKDDLESIK	KVIKEEKEKF	P-----	SSPPT	TPPSPAKT--
UPA-p42	GISYIEKVL	KYKDDLESIK	KVIKEEKEKF	P-----	SSPPT	TPPSPAKT--
FC27-42	GISYIEKVL	KYKDDLESIK	KVIKEEKEKF	P-----	SSPPT	TPPSPAKT--
3D7-42	GISYIEKVL	KYKDDLESIK	KVIKEEKEKF	P-----	SSPPT	TPPSPAKT--
Pv142	GVNYNKMGE	LYKTHLTAVN	EEVKKVEADI	KAEDDKIKKI	GSDDST	KTEKTOS--
Pv242	GVTYNKMGE	LYKTHLDGK	TEIKKVEDDI	KKQDEELKKL	GNVNS	QDSKKNF--
Pcyn42	ELAYYQKMG	LYKKHLDEVN	AQIKEVEANI	NKHDEEIKKI	GSEAS	KANDKNOL--
Pk42	EMEYYKKMG	LYKQHLEAVN	AQIKEIEASV	P-----		--GEQS@L--
Py42	EIKFFNKME	LYNTOLA AVK	EQIATIE---		A	ETNDTN---
Pb42	EIKFFNKME	LYKIOLA AVK	EQIDAIE---		A	ATTD-----
Pc42	EIEFFNKME	LYKPOLNAVN	EQIAAIG---			EPTDAEK---

190 200 210 220 230 240

Pfwel42		DKQGE	-NEKYLFELN	NIETLYKTVN	DKIDLFVIL	EAKVLNYYTE
PfMAD42		DEQKK	-ESKFLFELT	NIETLYNNLV	NKIDDYLINL	RAKINDCNVE
CAMP-42		DEQKK	-ESKFLFELT	NIETLYNNLV	NKIDDYLINL	RAKINDCNVE
UPA-p42		DEQKK	-ESKFLFELT	NIETLYNNLV	NKIDDYLINL	RAKINDCNVE
FC27-42		DEQKK	-ESKFLFELT	NIETLYNNLV	NKIDDYLINL	RAKINDCNVE
3D7-42		DEQKK	-ESKFLFELT	NIETLYNNLV	NKIDDYLINL	RAKINDCNVE
Pv142		MAKKA	ELEKYLPELN	SIOKEYESLV	SKVNTYTDNL	KKVINNCOLE
Pv242		IAKKA	ELEKYLPELN	SIOKEYESLV	SKVNTYTDNL	KKVINNCOLE
Pcyn42		NAKKE	ELQKYLPELN	SIOKEYSTLV	NKVHSYTDNL	KKIINNCOLE
Pk42		NAQKE	ELKKYLPELN	SIOKEYESLV	NMAHTYKENL	KKFINNCOLE
Py42	-----K	E-----	EKK	---KYIEILE	DLKGLYETVI	GQAEFFYSEEL
Pb42	-----K	D-----	EKK	---KYVEIFE	DLKGLYETIL	GQSEFFYIEVL
Pc42		-----K	---	KYAEIFE	DLKGLYETIL	NGAEFFSELL

250                      260                      270                      280                      290                      300

Pfwel42	KSNVEVKIKE	LNLYKTIQDK	LADEKKNNNF	VGIADLSTDY	NHNNLTKEFL	STGMVFENLA
PfMAD	KDEAHVKITK	LSDLKAIDDK	IDLEKNTNDF	EAIKKLIND	TKKDMLGKLL	STGLV-QNFP
CAMP-42	KDEAHVKITK	LSDLKAIDDK	IDLEKNNHDF	EAIKKLIND	TKKDMLGKLL	STGLVQN-FP
UPA-p42	KDEAHVKITK	LSDLKAIDDK	IDLEKNNHDF	DAIKKLIND	TKKDMLGKLL	STGLVQN-FP
FC27-42	KDEAHVKITK	LSDLKAIDDK	IDLEKNTNDF	EAIKKLIND	TKKDMLGKLL	STGLVQN-FP
3D7-42	KDEAHVKITK	LSDLKAIDDK	IDLEKNPYDF	EAIKKLIND	TKKDMLGKLL	STGLVQN-FP
Pv142	KKEAEITVKK	LQDYNKMDK	LEEYKKS---	-----EKKNEV	KSSGLEKLM	KSKLIKENES
Pv242	KKEAEITVKK	LQDYNKMDK	LEEYKKS---	-----EKKNEV	KSSGLEKLM	KSKLIKENES
Pcyn42	KKETETIVKK	LEDYSKMDEE	LDVYKOS---	-----KKEDDV	KSSGLEKLM	NSKLINQEEES
Pk42	KKETETIVKK	LEDYTKIDEN	LEIYKKS---	-----KKESDV	RSSGLEKLIK	NSKLINEEVS
Py42	KAEFEILTGN	LEKYIQIDDK	LDEEVEHAE-	-----NNKH	IASIALNNLN	KSELVGESES
Pb42	KTEFEILTGN	LETYIKIDDK	LENEVEN---	-----AENNKH	IASIALNNLN	KSELVGESES
Pc42	KAGFDILMAN	LETYIRIDDK	LHDEVEHAE-	-----KNKH	IASIALNNLN	KSELVTESES

310                      320                      330                      340                      350                      360

Pfwel42	KTVLSNLLDG	NLQGMNLIS-	QOCVKK-QC	PENSECFRIL	DEREECKCLL	NYK--QEGDK
PfMAD42	NTLISKLLIEG	KFODMLNIS-	QOCVKK-QC	PENSECFRIL	DEREECKCLL	NYK--QEGDK
CAMP-42	NTLISKLLIEG	KFODMLNIS-	QOCVKK-QC	PENSECFRIL	DEREECKCLL	NYK--QEGDK
UPA-p42	NTLISKLLIEG	KFODMLNIS-	QOCVKK-QC	PENSECFRIL	DEREECKCLL	NYK--QEGDK
FC27-42	NTLISKLLIEG	KFODMLNIS-	QOCVKK-QC	PENSECFRIL	DEREECKCLL	NYK--QEGDK
3D7-42	NTLISKLLIEG	KFODMLNIS-	QOCVKK-QC	PENSECFRIL	DEREECKCLL	NYK--QEGDK
Pv142	KEILSOLLNV	QTCLLTMS-	ETCICDT-NV	PENACACRYL	DGMEEWRCCL	TFK--EKGK
Pv242	KEILSOLLNV	QTCLLTMS-	ETCICDT-NV	PENACACRYL	DGTEEWRCLL	TFK--EKGK
Pcyn42	KKALSOLLNV	QTCLLTMS-	ETCICDT-NV	PENACACRYL	DGTEEWRCLL	YFK--EDAGK
Pk42	KKVLSOLLNV	QTCLLTMS-	ETCICDT-NV	PENACACRYL	DGTEEWRCLL	GEK--EKGK
Py42	KKILAKMLNM	DGMDLIGVDP	KEVCVDTTRDI	KNACCFRDD	NGTEEWRCLL	GYKKG--NT
Pb42	KKILAKMLNM	DSMDLIGVDP	KEVCINTRDI	PANACCFRYD	NGNEEWRCLL	GYKKN--NT
Pc42	KKILAKMLNM	DAMDILIGGS	NHVCIST-ST	PANACCFRYD	DGTEEWRCLL	GEKCKDDNR

370                      380                      390                      400                      410                      420

Pfwel42	CVENPNPTCN	ENNGCCDADA	KCTEEDSGSN	--GKKITCEC	TKEDSYPLFD	GIFCSSSNFL
PfMAD42	CVENPNPTCN	ENNGCCDADA	TCTEEDSGSS	--RKKITCEC	TKEDSYPLFD	GIFCSSSNFL
CAMP-42	CVENPNPTCN	ENNGCCDADA	KCTEEDSGSN	--GKKITCEC	TKEDSYPLFD	GIFCSSSNFL
UPA-p42	CVENPNPTCN	ENNGCCDADA	KCTEEDSGSN	--GKKITCEC	TKEDSYPLFD	GIFCSSSNFL
FC27-42	CVENPNPTCN	ENNGCCDADA	TCTEEDSGSS	--RKKITCEC	TKEDSYPLFD	GIFCSSSNFL
3D7-42	CVENPNPTCN	ENNGCCDADA	TCTEEDSGSS	--RKKITCEC	TKEDSYPLFD	GIFCSSSNFL
Pv142	CVPASNVTCK	DNNECCAPEA	ECKMTDSN--	----KIVCKC	TKEGSEPLFE	GVFCSSSSFL
Pv242	CVPASNVTCK	DNNECCAPEA	ECKMTDSN--	----KIVCKC	TKEGSEPLFE	GVFCSSSSFL
Pcyn42	CVPAPNMTC	DNNECCAPEA	ECKMND----	--KNEIVCKC	TKEGSEPLFE	GVFCSSSSFL
Pk42	CVPAS-ITCE	ENNGCCAPEA	ECTMED----	--KKEVECKC	TKEGSEPLFE	GVFCSSSSFL
Py42	CVENNNPTCD	INNGCCDPTA	SCQNAESTEN	--SKKIITC	KEPTPNAYYE	GVFCSSSSFL
Pb42	CTEDSNPTCG	NNNGCCDPTA	GCQTAENREN	--SKKIITC	KEPTPNAYYE	GVFCSSSSFL
Pc42	CVADDAEVCN	NNNGCCDPTA	DREVENTDR	DPSKKIVCTC	KEENPNAYYA	GVFCSSSSFL

430

Pfwel42	GISFLLILML	ILYSFI-
PfMAD42	GISFLLILML	ILYSFI-
CAMP-42	GISFLLILML	ILYSFI-
UPA-p42	GISFLLILML	ILYSFI-
FC27-42	GISFLLILML	ILYSFI-
3D7-42	GISFLLILML	ILYSFI-
Pv142	SLSFLLILML	FLLCMEL
Pv242	SLSFLLILML	FLLCMEL
Pcyn42	-----	-----
Pk42	-----	-----
Py42	GLSIHLLITL	IVFNIF-
Pb42	GLSIHLLITL	IVFNIF-
Pc42	GLSIHLLITL	IVFNLF-

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